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Role of surface charge heterogeneity in Enterococcus faecalis adhesion and biofilm formation
van Merode, Annet

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Enterococcus faecalis is a commensal bacterium found in the gut of most animal species. It is also a leading cause of nosocomial infections in humans and has the ability to adhere to the surface of biomaterials and form biofilms on them. Biliary stents are frequently colonized by bacteria, which form biofilms in the stents leading to clogging of the stent. One of the predominant strains isolated from clogged biliary stents is *E. faecalis*.

Several enterococcal virulence factors as the enterococcal surface protein, gelatinase, and aggregation substances, which are reported to be involved in adhesion and biofilm formation are described in **Chapter 1**. Further, the role of physicochemical cell surface properties (cell surface charge and hydrophobicity), determined by the composition of the cell envelope, in adhesion are discussed. The main aim of this thesis is to study the culture heterogeneity in zeta potential, its mechanism and its effect on adhesion and biofilm formation by *E. faecalis* strains isolated from clogged biliary stents.

In **Chapter 2**, six *E. faecalis* strains isolated from clogged biliary stents were investigated for the presence of specific biochemical factors involved in their adhesion: aggregation substances (Agg) and the enterococcal surface protein (encoded by the *esp* gene). In addition, physicochemical factors involved in adhesion (zeta potential and cell surface hydrophobicity) were determined, as well as the influence of ox bile on these properties. Two third of the biliary stent isolates displayed culture heterogeneity in the pH dependence of their zeta potentials. Moreover, 24 out of 46 clinical isolates of *E. faecalis*, including 11 laboratory strains, also displayed this heterogeneity. The culture heterogeneity was demonstrated to be a stable trait, not caused by quorum sensing, not plasmid mediated, and independent of the presence of *esp* and Agg. Data presented in Chapter 2 show that culture heterogeneity in zeta potential enhances adhesion to an abiotic surface. A higher prevalence of culture heterogeneity in zeta potential in pathogenic as compared to non-pathogenic isolates could indicate that this phenomenon might serve a role in virulence and putatively in pathogenesis.

The influence of culture heterogeneity on initial adhesion and subsequent biofilm formation was investigated in **Chapter 3** using a microtiter plate biofilm assay. Heterogeneous strains were retained in higher numbers on polystyrene

Summary

than homogeneous strains. Also, biofilm formation was much more pronounced for heterogeneous strains than for homogeneous strains. In a population, enriched to display only one subpopulation, fewer bacteria were retained than in its original heterogeneous culture. Furthermore, this enriched subpopulation formed less biofilm than its original heterogeneous culture. The presence of ox bile during adhesion resulted in fewer retained bacteria, although heterogeneous strains were still retained in significantly higher numbers than were homogeneous strains, and, in general, the presence of ox bile reduced biofilm formation. The initial adhesion and biofilm formation were independent of the presence of the *esp* gene or the expression of gelatinase.

Although it is clear that heterogeneous strains adhere in higher numbers, it is unclear in which stage of the adhesion process heterogeneity is involved: in the initial contact between bacteria and a surface or after some time of adhesion when adhered bacteria influence the adhesion of other adhering bacteria. Therefore, in **Chapter 4** the initial adhesion kinetics of five heterogeneous and five homogeneous *E. faecalis* strains were compared. It was shown that after 4 h of flow, heterogeneous strains adhered in significantly higher numbers than homogeneous strains (7.3×10^6 and 1.9×10^6 cm⁻², respectively), but the initial deposition rates were not significantly influenced (740 and 600 cm⁻² s⁻¹, respectively). Heterogeneous strains become homogeneous upon growth in the presence of ox bile, as also shown in Chapter 2, and initial adhesion of the individual strains comprising this group decreased. Yet, growth in the presence of ox bile, did not significantly affect the group averaged initial deposition rates or the numbers of bacteria adhering after 4 h, neither for the heterogeneous nor for the homogeneous strains. Apparently, initial deposition of bacteria is mainly governed by attractive Lifshitz-Van der Waals forces that out-compete the electrostatic repulsion, thus resulting in similar initial deposition rates for both groups. In contrast, during later stages, bacteria in heterogeneous cultures will experience less electrostatic repulsion from already adhering bacteria than bacteria in homogeneous cultures allowing closer proximity of the bacteria, resulting in an increased 4 h adhesion. Based on the results as reported in Chapter 3 and 4, it is concluded that heterogeneity in cell surface charge represents an advantage for bacteria in the colonization of surfaces.

The observations that homogeneous *E. faecalis* are unable to form biofilm raised the question of how these had been able to grow in a biofilm on clogged biliary stents. In **Chapter 5**, the influence of 15 other microbial strains isolated from clogged biliary stents on the prevalence of *E. faecalis* strains in mixed species biofilms was investigated. In general, the prevalence of the charge-heterogeneous, biofilm forming *E. faecalis* strains was reduced in mixed species biofilms. The prevalence of charge-homogeneous *E. faecalis* strains lacking the ability to form biofilms on their own, was increased only in the presence of *Citrobacter freundii* BS5126, *Stenotrophomonas maltophilia* BS937, and *Candida lusitanae* BS8256 all of which introduced a sizeable charge heterogeneity among the mixed bacterial population in the biofilm.

The ability of microorganisms to form biofilm is often examined using a microtiter plate biofilm assay, as used in Chapters 3 and 5. Microtiter plate biofilm assays are used because of their simplicity and efficiency. However, different laboratories apply the assay in slightly different ways, which are unfortunately crucial to the results and conclusions drawn. In **Chapter 6**, two steps in the microtiter plate biofilm assay are evaluated. First, it is shown that the solubilization of the retained stain by the biofilms leads to different conclusions on the ability of a series of biliary stent isolates to form biofilm. Secondly, it is demonstrated that the explicit inclusion of initial adhesion in the assay, as the first step in biofilm formation, yields more extensive enterococcal biofilms than when biofilms were grown directly from a suspension in growth medium.

The results of this thesis are discussed in **Chapter 7**. This thesis shows that heterogeneity in zeta potential is a common phenomenon among *E. faecalis* strains and forms an advantage in the colonization of surfaces. However, the molecular basis and the processes that regulate this phenomenon have to be identified to fully understand its role in virulence and adhesion.

Annet van Merode, 2006